# Procedure & Checklist – No-Amp Targeted Sequencing Utilizing the CRISPR-Cas9 System

## Before You Begin

This procedure describes sequencing targeted genomic regions, without amplification, on the PacBio® Sequel® System by using a CRISPR-Cas9 enrichment methodology. Genomic DNA (gDNA) is first treated to prevent any termini arising during fragmentation from participating in SMRTbell® library preparation. The treated gDNA is subsequently subjected to targeted CRISPR-Cas9 digestion.

Cas9 nuclease, in association with two individual guide RNA (gRNA) oligonucleotides, identifies and then cleaves a specific recognition site on each side of the target region. Different gDNA regions can be targeted in a single CRISPR-Cas9 digest reaction using multiple gRNA pairs. After RNA-directed cleavage, adapters are ligated to the blunt template ends, forming SMRTbell templates. In the final step, unligated DNA is eliminated by exonuclease digestion.

Note that this procedure is to be used in conjunction with customer-designed assays. Assay design involves generating and selecting CRISPR-Cas9 RNA oligonucleotides that will excise a target region of interest. CRISPR-Cas9 RNA oligos are ordered through a third-party vendor.

This document presents an example No-Amp targeted sequencing workflow that has been tested and validated at PacBio for analyzing the following genomic regions: the CAG repeat locus at the 5' end of the *HTT* gene (4p16.3), the CGG repeat locus in the 5' UTR of the *FMR1* gene (Xq27.3), the ATTCT repeat locus in intron 9 of the *ATXN10* gene (22q13.31), and the intronic GGGGCC repeat locus of the *C9orf72* gene (9p21.2). Ordering information for RNA oligos specific to these four targets is listed in the **Additional Required Materials** section. Technical guidance on design of CRISPR-Cas9 RNA oligonucleotides for other targets is provided in *Reference Guide – Designing CRISPR-Cas9 RNA Oligonucleotides for the No-Amp Targeted Sequencing Procedure*.

Sequel II System compatibility is not fully supported with this procedure. Please contact customer support at support@pacb.com or your local field application scientist for inquiries on running this procedure on the Sequel II System.

## DNA Input Requirements

The starting gDNA input amount must be between 5 to 10 µg represented by either a single sample or the total of multiple samples that will be multiplexed. This range of input amount will typically result in 25 to 200 ng of final SMRTbell library.

Multiplexing up to 10 samples is possible with a minimum starting input of 1 µg gDNA per sample.

The entire SMRTbell library (either one or several samples) is loaded onto a single SMRT Cell.

We recommend measuring the amount of double-stranded DNA using a fluorometric quantitation method such as the Qubit® Quantitation Platform.

## Evaluate gDNA Quality

Optimal results are achieved with high-purity, high-molecular-weight gDNA. Before starting this procedure, we recommend evaluating gDNA size and integrity using a pulsed-field electrophoresis system. Any of the three commercially available systems and methods listed in Table 2 below may be used to evaluate gDNA quality.

Table 2. Genomic DNA quality evaluation methods.

| Method | Procedure/Product Note |
|---|---|
| Bio-Rad® CHEF Mapper® XA Pulsed Field Electrophoresis System | Procedure & Checklist - Using the BIO-RAD® CHEF Mapper® XA Pulsed Field Electrophoresis System |
| Sage Science™ Pippin Pulse Electrophoresis Power Supply | Procedure & Checklist - Using the Sage Science™ Pippin Pulse Electrophoresis Power Supply System |
| Agilent FEMTO Pulse™ System | Product Note - Fast, High-Resolution DNA Sizing with the Agilent Femto Pulse System |

For best results, ensure the fragment size mode for a gDNA sample is ≥ 50 kilobase pairs (see Figure 1 below). For gDNA samples with fragment size mode < 50 kb, increasing gDNA input above 10 µg can help increase the yield of on-target reads but may require reaction condition adjustments.

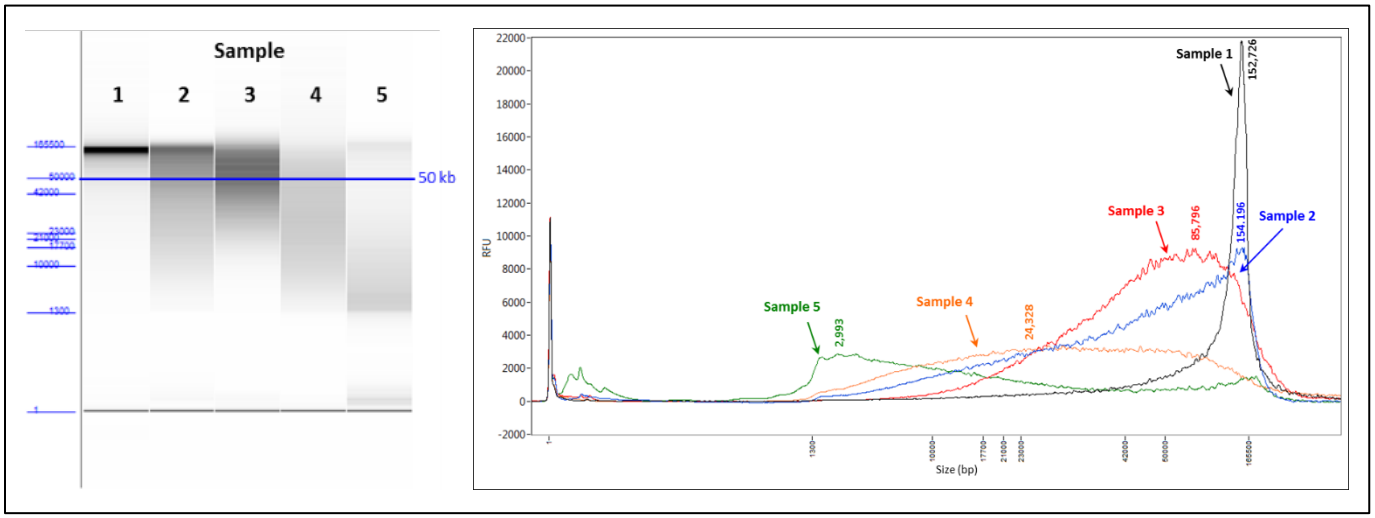


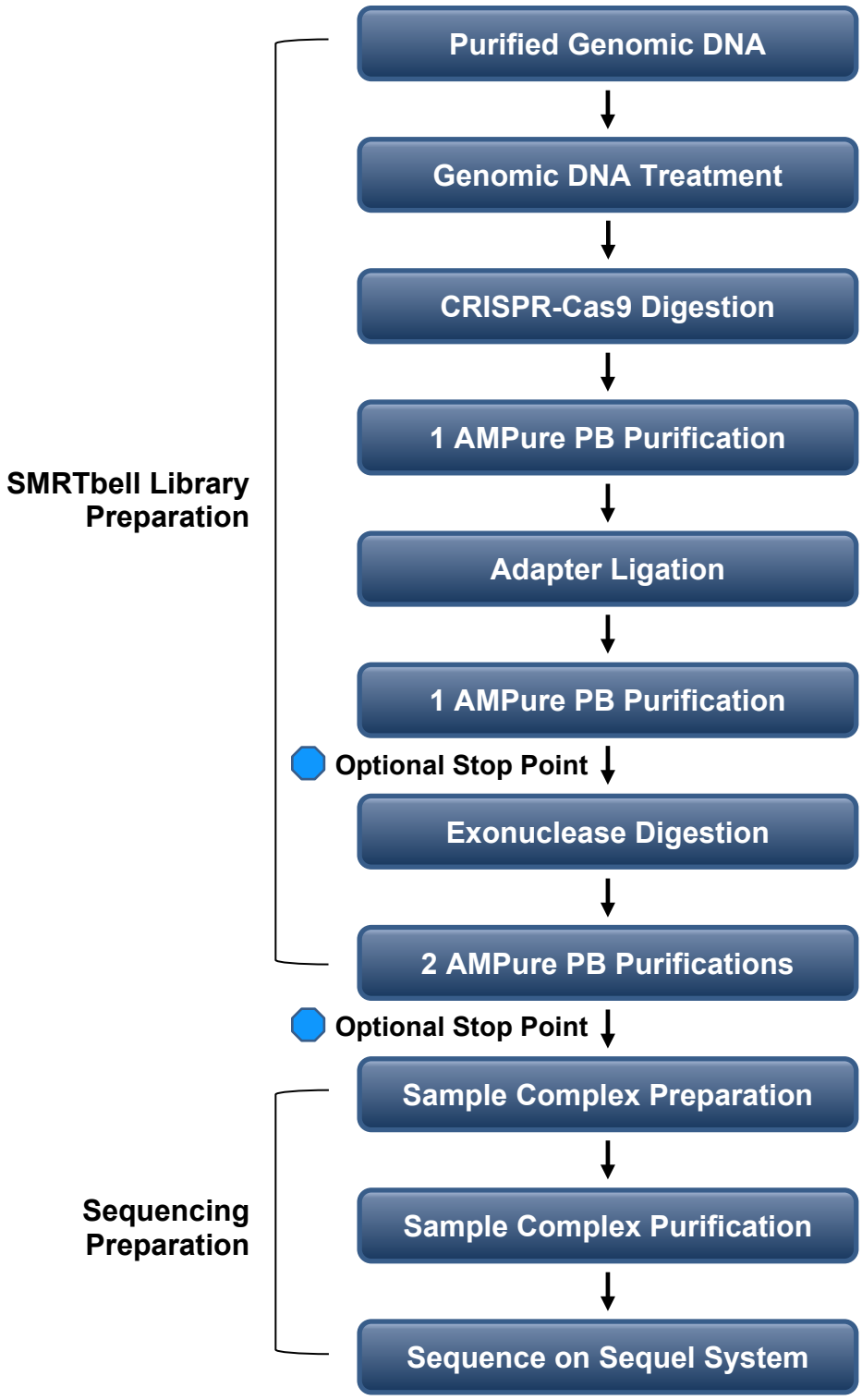

Figure 1. Evaluation of gDNA quality using FEMTO Pulse. Samples 1 through 5 depict a wide degree of DNA integrity with various amounts of degradation. The majority of fragments for Samples 1, 2 and 3 are above 50 kb while those for Sample 4 and 5 are below 50 kb. While increased gDNA input may generate on-target reads for Sample 4 comparable to Samples 1, 2 and 3, the level of degradation for Sample 5 is such that very few intact target molecules may be obtained with the maximum amount of gDNA input of 10 µg.

Assessment of gDNA purity can be achieved by examining the 260 nm to 280 nm ($OD_{260/280}$) and 260 nm to 230 nm ($OD_{260/230}$) absorbance ratios obtained by spectrophotometric analysis. Genomic DNA should have $OD_{260/280}$ and $OD_{260/230}$ ratios of 1.8-2.0 and 2.0-2.2, respectively. Superior performance may be achieved by re-purifying gDNA samples with AMPure® PB reagent if $OD_{260/280}$ and $OD_{260/230}$ ratios fall substantially outside the ideal value ranges.

## Best Practices for Generating No-Amp Targeted Sequencing Results

1. Use the required DNA input amount specified in the **DNA Input Requirements** section.
2. Use DNA extraction methods optimized for the gDNA source material.
3. Maintain enzymes at -20°C in a benchtop cooler during reaction setup. Return enzymes to storage once reaction setup is complete.
4. Minimize tube transfers to reduce the amount of sample lost on tube walls and pipette tips.
    a. Prepare and incubate reactions and perform AMPure® PB purifications in tubes of the same size whenever possible.
5. Ensure reactions are adequately mixed prior to incubation.
6. Use dedicated nuclease-free water and consumables when working with RNA oligos. Keep RNA oligos and associated reagents for CRISPR-Cas9 digestion on ice at all times.
7. Allow sufficient time for AMPure PB reagent and Elution Buffer to reach room temperature before use.
8. When performing sequential AMPure PB bead purifications, transferring a small number of beads from the first purification into the binding step of the second purification is preferred over leaving some eluate behind to minimize sample loss.
9. Slowly remove supernatant and ethanol washes during AMPure PB bead purifications to minimize bead loss due to fluid motion.
10. Be mindful not to over-dry AMPure PB beads during purification steps.

## Workflow


Purified Genomic DNA
Genomic DNA Treatment
CRISPR-Cas9 Digestion
1 AMPure PB Purification
Adapter Ligation
1 AMPure PB Purification
Optional Stop Point
Exonuclease Digestion
2 AMPure PB Purifications
Optional Stop Point
Sample Complex Preparation
Sample Complex Purification
Sequence on Sequel System
SMRTbell Library Preparation
Sequencing Preparation

# Required Materials

## Equipment and Consumables

| Item | Vendor | Part Number | Comments |
|---|---|---|---|
| 0.5 mL and 1.5 mL DNA LoBind Tubes | Eppendorf | 0030108035 and 0030108051 | |
| 0.2 mL PCR Thermal Cycling Tubes | USA Scientific | various | |
| Molecular Biology Pipettes, Standard Set | MLS | - | capable of pipetting volumes 0.5 µL to 1 mL |
| Wide-bore Pipette Tips with Filter | Rainin Instrument | 30389241 | |
| Benchtop Cooler | MLS | - | for maintaining enzymes at -20°C |
| Microcentrifuge | MLS | - | 14,000 RCF capability |
| Mini Centrifuge | MLS | - | for quick spins |
| Vortex Mixer | MLS | - | 2,000 rpm capability |
| DynaMag™-2 Magnet | Thermo Fisher Scientific | 12321D | 16 x 1.5-2.0 mL microcentrifuge tubes |
| MagneSphere® Technology Magnetic Separation Stand (optional) | Promega | Z5341 | 12 x 0.5 mL microcentrifuge tubes; optional magnetic stand |
| Neodymium Disc Magnet, 1/4” dia. by 1/8” thick (optional) | K&J Magnetics | D42-N52 | used with stand Z5341 listed above |
| PCR Thermal Cycler | MLS | - | |
| ThermoMixer C with heated lid (or equivalent) | Eppendorf | 5382000023 | 15-100ºC range |
| Qubit® Quantitation Platform (Qubit 2.0, 3, or 4) | Thermo Fisher Scientific | various | |
| Qubit™ 1X dsDNA HS Assay Kit | Thermo Fisher Scientific | Q33230 | |
| SMRT Cell 1M v3 Tray (4 cells) or SMRT Cell 1M v3 LR Tray (4 cells) | PacBio | 101-531-000 or 101-531-001 | 10-hr max collection time or 20-hr max collection time |
| Sample Plate (50 plates) | PacBio | 000-448-888 | 50 plates per case |
| Sequel Sample Plate Foil | PacBio | 100-667-400 | 100 foil seals per case |
| Sequel Mixing Plate (120 Pack) | PacBio | 100-667-500 | 120 plates per case |
| Tube Septa | PacBio | 100-667-700 | 10 tube septa per bag |
| Sequel Pipet Tips v2 (40 racks / case) | PacBio | 100-667-601 | 40 racks of 96 tips each per case |

## Reagents – Third-Party Vendors

| Item | Vendor | Part Number | Amount Provided |
|---|---|---|---|
| Nuclease-Free Water, not DPEC-Treated | Ambion | AM9937 | 10 x 50 mL |
| IDTE pH 7.5 Buffer (1X TE Solution) | IDT | 11-01-02-02 or 11-05-01-15 | 10 x 2 mL or 300 mL |
| Nuclease-Free Duplex Buffer | IDT | 11-01-03-01 or 11-05-01-12 | 10 x 2 mL or 300 mL |
| Ethyl Alcohol, Molecular Biology Grade | MLS | - | |
| 0.5 M EDTA, pH 8.0, Molecular Biology Grade | MLS | - | |
| Shrimp Alkaline Phosphatase (rSAP) | New England BioLabs | M0371S or M0371L | 500 or 2,500 units @ 1,000 U/mL |
| Klenow Fragment (3'→5' exo-) | New England BioLabs | M0212S or M0212L | 200 or 1,000 units @ 5,000 U/mL |
| Dideoxynucleoside Triphosphate Set, Sequencing Grade, Sodium Salt | Sigma-Aldrich | 3732738001 | 100 µL each ddATP, ddCTP, ddGTP and ddTTP @ 10 mM |
| CutSmart® Buffer, 10X | New England BioLabs | B7204S | 5 mL each |
| Exonuclease III | New England BioLabs | M0206S or M0206L | 5,000 or 25,000 units @ 100,000 U/mL |
| Exonuclease VII | New England BioLabs | M0379S or M0379L | 200 or 1,000 units @ 10,000 U/mL |
| Cas9 Nuclease, *S. pyogenes* | New England BioLabs | M0386T or M0386M | 400 or 2,000 pmol @ 20 µM |
| NEBuffer™ 3.1[1], 10X | New England BioLabs | B7203S | 5 mL each |
| T4 DNA Ligase Reaction Buffer, 10X | New England BioLabs | B0202S | 6 mL each |
| T4 DNA Ligase, HC | Thermo Fisher Scientific | EL0013 | 5000 Weiss units @ 30 U/µL |
| SOLu-Trypsin | Sigma-Aldrich | EMS0004 | 100 µg @ 1 mg/mL |

[1]NEBuffer™ 3.1, 10X is also included with NEB Cas9 Nuclease, *S. pyogenes*.

## Reagents - PacBio

| Item | Vendor | Part Number | Amount Provided |
|---|---|---|---|
| Sequel Binding and Internal Control Kit 3.0 | PacBio | 101-626-600 | |
| Sequel Sequencing Kit 3.0 (4 rxn) or Sequel Sequencing Kit 3.0 (8 rxn) | PacBio | 101-597-900 or 101-597-800 | 4-rxn kit or 8-rxn kit |
| Sequel SMRT Cell Oil | PacBio | 100-621-300 | 5 tubes, 1600 µL each |
| AMPure PB | PacBio | 100-265-900 | 1 bottle, 5 mL |
| Elution Buffer | PacBio | 101-633-500 | 1 bottle, 50 mL |
| No-Amp Accessory Kit, containing: | PacBio | 101-788-900 | |
| – Sequencing Primer v4 | PacBio | - | 1 tube, 18 µL |
| – 10X Primer Buffer v2 | PacBio | - | 1 tube, 1000 µL |
| – 10X Annealing Buffer | PacBio | - | 2 tubes, 1000 µL each |

# Additional Required Materials

### CRISPR-Cas9 RNA Oligonucleotides

In addition to the materials listed above, CRISPR-Cas9 RNA oligos that are compatible with customer-designed assays are required. Refer to *Reference Guide – Designing CRISPR-Cas9 RNA Oligonucleotides for the No-Amp Targeted Sequencing Procedure* for technical guidance on designing target-specific oligos.

The following RNA oligos are used for assays targeting HTT, FMR1, ATXN10, and C9orf72 repeat elements in the human genome.

| Item | Sequence (5'-3') | Vendor | Part Number | Amount Provided |
|---|---|---|---|---|
| Alt-R® CRISPR-Cas9 crRNA, HTT repeat element target, "HTT.DC.1" | CTTATTAACAGCAGAGAACT | IDT | - | 2 or 10 nmol scale |
| Alt-R® CRISPR-Cas9 crRNA, HTT repeat element target, "HTT.DC.2" | TAAACTTTGAAGACGAGACA | IDT | - | 2 or 10 nmol scale |
| Alt-R® CRISPR-Cas9 crRNA, FMR1 repeat element target, "FMR1.DC.1" | CGCGCGTCTGTCTTTCGACC | IDT | - | 2 or 10 nmol scale |
| Alt-R® CRISPR-Cas9 crRNA, FMR1 repeat element target, "FMR1.DC.2" | CCTTTATGCAAAGTTAGCTC | IDT | - | 2 or 10 nmol scale |
| Alt-R® CRISPR-Cas9 crRNA, ATXN10 repeat element target, "ATXN.DC.1" | TGTTCCACCAGCCTTTGCCA | IDT | - | 2 or 10 nmol scale |
| Alt-R® CRISPR-Cas9 crRNA, ATXN10 repeat element target, "ATXN.DC.2" | TAAATTTCACCTGATCAAGG | IDT | - | 2 or 10 nmol scale |
| Alt-R® CRISPR-Cas9 crRNA, C9orf72 repeat element target, "C9orf72.DC.1" | TTGGTATTTAGAAAGGTGGT | IDT | - | 2 or 10 nmol scale |
| Alt-R® CRISPR-Cas9 crRNA, C9orf72 repeat element target, "C9orf72.DC.2" | GGAAGAAAGAATTGCAATTA | IDT | - | 2 or 10 nmol scale |
| Alt-R® CRISPR-Cas9 tracrRNA | universal sequence provided by IDT | IDT | 1072532, 1072533 or 1072534 | 5, 20 or 100 nmol scale |

### PacBio Barcoded Adapters

PacBio Barcoded Adapters listed in the table below must be ordered from a third-party vendor (such as IDT). They are compatible with any assay designed for this procedure. In addition to this subset of 16 barcoded adapters, the following link contains ordering information for the full set of 384 barcoded adapters: Symmetric Barcoded Adapters Ordering Sheet for 384 barcodes.

Adapters require a 5' phosphorylation modification as indicated in the table below. HPLC purification is recommended.

| Item | Sequence (5'-3') | Vendor |
|---|---|---|
| Barcoded Adapter bc1001 | /5Phos/CGCACTCTGATATGTGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAG AGATCACATATCAGAGTGCG | IDT |
| Barcoded Adapter bc1002 | /5Phos/CTCACAGTCTGTGTGTATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAG AGATACACACAGACTGTGAG | |
| Barcoded Adapter bc1004 | /5Phos/CGCGCGTGTGTGCGTGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAG AGATCACGCACACACGCGCG | |
| Barcoded Adapter bc1008 | /5Phos/CGCAGCGCTCGACTGTATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAG AGATACAGTCGAGCGCTGCG | |
| Barcoded Adapter bc1009 | /5Phos/TCTGTCTCGCGTGTGTATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAG AGATACACACGCGAGACAGA | |
| Barcoded Adapter bc1010 | /5Phos/CTCTGAGATAGCGCGTATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAG AGATACGCGCTATCTCAGAG | |
| Barcoded Adapter bc1012 | /5Phos/ACACGCGATCTAGTGTATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAG AGATACACTAGATCGCGTGT | |
| Barcoded Adapter bc1014 | /5Phos/ACGCGCGCGTAGTGAGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAG AGATCTCACTACGCGCGCGT | |
| Barcoded Adapter bc1015 | /5Phos/ACACACGTGTCATGCGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAG AGATCGCATGACACGTGTGT | |
| Barcoded Adapter bc1016 | /5Phos/ATACTATCTCTCTATGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAG AGATCATAGAGAGATAGTAT | |
| Barcoded Adapter bc1017 | /5Phos/ATATAGCGCGCGTGTGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAG AGATCACACGCGCGCTATAT | |
| Barcoded Adapter bc1018 | /5Phos/CACAGTGAGCACGTGAATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAG AGATTCACGTGCTCACTGTG | |
| Barcoded Adapter bc1019 | /5Phos/ATCTGATAGAGTGTGTATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAG AGATACACACTCTATCAGAT | |
| Barcoded Adapter bc1020 | /5Phos/ACATCGTCGTGTCGTGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAG AGATCACGACACGACGATGT | |
| Barcoded Adapter bc1021 | /5Phos/ACATCACTATGTATAGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAG AGATCTATACATAGTGATGT | |
| Barcoded Adapter bc1022 | /5Phos/ATATCACACGTGAGTGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAG AGATCACTCACGTGTGATAT | |

# Reagent Preparation

### CRISPR-Cas9 RNA Oligonucleotides

Prepared 50 µM stocks of crRNA and tracrRNA oligos are used to create gRNA in the **Guide RNA Preparation** step. Guide RNA is subsequently used in the **Cas9 Digestion** step.

- Resuspend each crRNA and tracrRNA oligo separately in nuclease-free IDTE pH 7.5 buffer (10 mM Tris, 0.1 mM EDTA) to a final concentration of 50 µM.
  **Note:** Accuracy of the final concentration can be improved by targeting a higher initial concentration (10-20% higher) and adjusting down to the final concentration using the measured concentration of the initial resuspension.
- Aliquot to individual tubes for a maximum of 10 uses per tube to minimize freeze/thaw cycles.
- Store all 50 µM RNA oligo preparations at -80°C.

### PacBio Barcoded Adapters

Prepared 20 µM stocks of Barcoded Adapters are used in the **Adapter Ligation** step.

**Note:** Annealed working stocks can be prepared in advance and stored without requiring additional reannealing.

- Resuspend adapters in nuclease-free IDTE pH 7.5 buffer (10 mM Tris, 0.1 mM EDTA) to 100 µM.
- Prepare 20 µM working stocks in 1X Annealing Buffer (supplied at 10X concentration from PacBio in the No-Amp Accessory Kit) and nuclease-free water (molecular biology grade).
  **Note:** The final concentration of Annealing Buffer in the 20 µM adapter working stock solution is 1X.
  **Note:** Accuracy of the final concentration can be improved by targeting a higher initial working stock concentration (10-20% higher) and adjusting down to the final concentration using the measured concentration of the initial working stock.
- Anneal the 20 µM working stocks as follows: Incubate in a thermal cycler at 80°C for 5 minutes then ramp to 25°C at a rate of 0.1°C per second. Hold at 4°C.
- Store all adapter preparations at -15 to -25°C.

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### ddNTP Mix

A 1 mM solution of ddATP, ddCTP, ddGTP and ddTTP is used in the **gDNA Treatment** step.

- Prepare a 1 mM mixture of ddATP, ddCTP, ddGTP and ddTTP (0.25 mM each ddNTP) in nuclease-free water.
- Aliquot to individual tubes for a maximum of 5 uses per tube to minimize freeze/thaw cycles.
- Store aliquots at -15 to -25°C.

## gDNA Treatment

Genomic DNA is first treated to prevent any fragment ends from participating in ligation following the **CRISPR-Cas9 Digestion** step. This reduces off-target molecules in the final SMRTbell library.

The required starting gDNA input amount for this procedure is 5 to 10 µg. For samples that will not be multiplexed, use 5 to 10 µg gDNA for this step. If samples are to be multiplexed, determine the sample input amount by dividing the desired total input amount by the number of samples to be multiplexed. A maximum of 10 samples can be multiplexed with a minimum of 1 µg gDNA per sample.

### Dephosphorylation Reaction Setup

Prepare a separate reaction for each sample. The reaction table below accommodates an input amount from 1 to 5 µg gDNA. For input greater than 5 µg up to 10 µg, increase the reaction volume to 138 µL and scale-up reagents proportionally to maintain buffer and enzyme concentrations listed in the table below. If sample concentration is too low, either concentrate the sample or scale-up the reaction volume, maintaining the final concentration of buffer and rSAP in the table.

1. To a LoBind microcentrifuge tube, add the following reagents in the order listed:

| Reagent | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|
| Genomic DNA | ____ ng/µL | ____ µL for ____ µg | ____ ng/µL | | |
| Water (nuclease-free) | | ____ µL to adjust to Total Volume | | | |
| NEBuffer 3.1 | 10X | 8.0 µL | 1.2X | | |
| rSAP | 1 U/µL | 1.5 µL | 0.022 U/µL | | |
| Total Volume | | 69.0 µL | | | |

2. Mix the reaction thoroughly by gently inverting several times or by slow pipette-mixing with a wide-bore tip.

   **Note:** To minimize DNA shearing, do not vortex or flick the tube.

3. Spin the tube briefly in a mini centrifuge to collect the liquid.
4. Incubate at 37ºC for 30 minutes and then either place on ice or immediately proceed to the next step.
5. Incubate at 65ºC for 5 minutes to inactivate the phosphatase and then place on ice.
6. Proceed to the next step.

### Klenow Fill-In Reaction Setup

The reaction table below accommodates an initial procedure input amount from 1 to 5 µg gDNA. For input greater than 5 µg up to 10 µg, increase the reaction volume to 160 µL and scale-up reagents proportionally to maintain reagent concentrations listed in the table below.

1. To the tube from the previous step, add the following reagents in the order listed:

   **Note:** ddNTP mix is prepared in advance of executing this procedure. Refer to the **Reagent Preparation** section.

| Reagent | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|
| gDNA, rSAP-treated | | 69.0 µL | | | |
| ddNTP mix | 1 mM | 8.0 µL | 0.10 mM | | |
| Klenow Fragment (3'→5' exo-) | 5 U/µL | 3.0 µL | 0.19 U/µL | | |
| Total Volume | | 80.0 µL | | | |

2. Mix the reaction thoroughly by gently inverting several times or by slow pipette-mixing with a wide-bore tip.

   **Note:** To minimize DNA shearing, do not vortex or flick the tube.

3. Spin the tube briefly in a mini centrifuge to collect the liquid.
4. Incubate at 37ºC for 30 minutes and then either place on ice or immediately proceed to the next step.
5. Incubate at 75ºC for 20 minutes to inactivate Klenow Fragment and then place on ice.
6. Proceed to the next step.

# CRISPR-Cas9 Digestion

### Guide RNA Preparation

Each of two crRNAs per target must be annealed to tracrRNA separately in a 1:1 ratio to form gRNAs before setting up the CRISPR-Cas9 reaction. An equimolar mixture of the two gRNAs complexed with Cas9 nuclease will identify and excise the targeted region from the gDNA. The stock concentration of gRNA used in the CRISPR-Cas9 reaction is 5 µM. When targeting multiple regions in the same CRISPR-Cas9 reaction, keep the total stock concentration of multiplexed gRNAs at 5 µM (see **Step 8** below).

**Note:** Dedicated nuclease-free water and consumables for working with RNA should be used in all steps using RNA oligonucleotides. Keep RNA oligos and associated reagents on ice at all times.

The total volume of gRNA prepared below (20.0 µL) is sufficient for Cas9 digestion of two samples with initial procedure input amounts from 1 to 5 µg gDNA or one sample with the initial procedure input amount greater than 5 µg up to 10 µg. Scale reagents proportionally for additional samples.

1. To a 0.2 mL PCR thermal cycling tube, add the following reagents:

   **Note:** crRNA 1 and crRNA 2 represent two crRNA oligos for a single assay design. The tracrRNA oligo is universal and not specific to any assay design.

| Reagent | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|
| crRNA 1 | 50 µM | 1.0 µL | 5 µM | | |
| tracrRNA | 50 µM | 1.0 µL | 5 µM | | |
| Nuclease-Free Duplex Buffer | | 8.0 µL | | | |
| Total Volume | | 10.0 µL | | | |

2. To a separate 0.2 mL PCR thermal cycling tube, add the following reagents:

| Reagent | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|
| crRNA 2 | 50 µM | 1.0 µL | 5 µM | | |
| tracrRNA | 50 µM | 1.0 µL | 5 µM | | |
| Nuclease-Free Duplex Buffer | | 8.0 µL | | | |
| Total Volume | | 10.0 µL | | | |

3. Mix the reactions well by pipetting or flicking the tubes.
4. Spin the tubes briefly in a mini centrifuge to collect the liquid.
5. Incubate the 1:1 mixtures of crRNA and tracrRNA at 95°C for 5 minutes in a thermal cycler.
6. Remove tubes to the benchtop and allow to cool to room temperature (15-25°C) for 5 minutes.
7. Place the gRNAs on ice after cooling to room temperature.
8. Combine the entire volume of both gRNA preparations to form the final 5 µM gRNA mixture for a single target. The gRNA is ready to use in single-target CRISPR-Cas9 digestions.

   **Note:** For multiple-target CRISPR-Cas9 digestions, combine equal volumes of target-specific gRNA mixtures in a LoBind microcentrifuge tube. When multiplexing gRNAs, maintain a final combined concentration of 5 µM.

9. Continue to the **Cas9 Digestion** step on the next page.

### Cas9 Digestion

First, a complex of gRNA and Cas9 nuclease is formed. The CRISPR-Cas9 digestion reaction is then initiated with the addition of treated gDNA.

The reaction table below accommodates an initial procedure input amount from 1 to 5 µg gDNA. For input greater than 5 µg up to 10 µg, increase the reaction volume to 200 µL and scale-up reagents proportionally to maintain reagent concentrations listed in the table below.

1. To a LoBind microcentrifuge tube, add the following reagents in the order listed:

| Reagent | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|
| NEBuffer 3.1 | 10X | 2.0 µL | 1X | | |
| gRNA (single- or multiple-target) | 5 µM | 8.0 µL | 400 nM | | |
| Cas9 Nuclease | 20 µM | 2.0 µL | 400 nM | | |
| Water (nuclease-free) | | 8.0 µL | | | |

2. Mix the reaction well by pipetting or flicking the tube.
3. Spin the tube briefly in a mini centrifuge to collect the liquid.
4. Incubate at 37ºC for 10 minutes and then place on ice.
5. Next, add the treated gDNA to the reaction tube from above as follows:

| Reagent | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|
| gDNA, treated | | 80.0 µL | | | |
| Total Volume | | 100.0 µL | | | |

6. Mix the reaction thoroughly by gently inverting several times or by slow pipette-mixing with a wide-bore tip.

   **Note:** To minimize DNA shearing, do not vortex or flick the tube.
7. Spin the tube briefly in a mini centrifuge to collect the liquid.
8. Incubate at 37ºC for 1 hour and then place on ice.
9. Immediately proceed to the next step.

### Post-Digestion

1. After incubation, add 5 µL 0.5 M EDTA per 100 µL reaction.
2. Mix the reaction well by pipetting or flicking the tube.
3. Spin the tube briefly in a mini centrifuge to collect the liquid and then return the tube to ice.
4. Proceed to the next step.

## AMPure PB Bead Purification of Cas9 Digest Products

Perform one purification following the **CRISPR-Cas9 Digestion** step using 0.45X sample volume of AMPure PB beads.

| STEP | ✓ | Instructions | Notes |
|---|---|---|---|
| **1** | | Add the appropriate volume of AMPure PB beads to the sample based on the specified ratio of beads to sample volume.<br><br>**Note:** Mix the bead reagent well until the solution appears homogeneous before dispensing. Pipette the reagent slowly as the bead mixture is viscous. Precise volumes are critical to the purification process. | |
| **2** | | Mix the solution thoroughly by vortexing or flicking the tube and then spin briefly to collect the liquid. | |
| **3** | | Vortex the tube for 10 minutes at 2,000 rpm to bind DNA to the beads. After vortexing, the solution should appear homogeneous.<br><br>**Note:** We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recovery. | |
| **4** | | Spin the tube briefly to collect the liquid. | |
| **5** | | Collect the beads to the side of the tube in a magnetic bead rack. Allow beads to separate for at least 2 minutes or until the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added. | |
| **6** | | With the tube still on the magnetic bead rack, slowly remove the cleared supernatant and save in another tube. Avoid disturbing the bead pellet.<br><br>**Note:** If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB purification steps to recover the DNA. | |
| **7** | | Wash beads with freshly prepared 80% ethanol.<br>– Do not remove the tube from the magnetic rack.<br>– Slowly dispense the 80% ethanol against the side of the tube opposite the beads, taking care not to disturb the bead pellet. Use a sufficient volume of 80% ethanol to fill the tube (400 µL for 0.5 mL tube or 1 mL for 1.5 mL tube).<br>– After 30 seconds, slowly remove the 80% ethanol and discard.<br><br>**Note:** 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Measure the volumes of water and ethanol separately before combining to ensure the solution is 80% ethanol. 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. | |
| **8** | | Repeat Step 7 above 2 more times for a total of 3 washes. | |
| **9** | | Remove residual 80% ethanol.<br>– Remove the tube from the magnetic bead rack and spin briefly to pellet the beads. Both the beads and any residual 80% ethanol will be at the bottom of the tube.<br>– Place the tube back on the magnetic bead rack. | |

| | | | |
|---|---|---|---|
| | | – Slowly remove any remaining 80% ethanol and discard. | |
| **10** | | Check for any remaining droplets in the tube. If droplets are present, repeat Step 9. | |
| **11** | | Remove the tube from the magnetic bead rack. Allow the beads to air-dry with tube caps open for 30 to 60 seconds. | |
| **12** | | Elute the DNA off the beads.<br>– Add 30 µL Elution Buffer to the beads and mix until homogeneous.<br>– Vortex the tube for 5 minutes at 2,000 rpm.<br>– Spin the tube briefly to collect the liquid and then place the tube back on the magnetic bead rack.<br>– Let the beads separate fully and then without disturbing the bead pellet, carefully transfer the supernatant containing DNA to a new LoBind microcentrifuge tube. Keep the purified DNA on ice.<br>– Discard the beads. | |
| **13** | | Verify your DNA amount and concentration using a Qubit fluorometer.<br>– Add 1 µL of eluted sample to 4 µL of Elution Buffer. Use 1 µL of this 5-fold dilution to measure the DNA concentration using the Qubit 1X dsDNA HS Assay Kit according to the manufacturer's recommendations.<br>– Record the concentration and amount of recovered sample. Typical DNA yield is between 70-90% of the starting gDNA input amount.<br>– Proceed to the next step. | |

## Adapter Ligation

Adapters are ligated to the purified products of CRISPR-Cas9 cleavage, creating a SMRTbell library with symmetric ends on the target molecules. For a multiplexed library, ligation reactions are pooled during post-ligation cleanup.

### Ligation Setup

Add the components below in the order listed. The reaction table below accommodates an initial procedure input amount from 1 to 10 µg gDNA.

**Note: Mix where instructed BEFORE adding ligase.**

1. To a LoBind microcentrifuge tube, add the following reagents in the order listed:

| Reagent | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|
| gDNA, Cas9-digested | | 29.0 µL | | | |
| Barcoded Adapter, annealed | 20 µM | 1.0 µL | 0.40 µM | | |
| **Mix before proceeding** | | | | | |
| T4 DNA Ligase Reaction Buffer | 10X | 5.0 µL | 1X | | |
| Water (nuclease-free) | | 13.5 µL | | | |
| **Mix before proceeding** | | | | | |
| T4 DNA Ligase | 30 U/µL | 1.5 µL | 0.90 U/µL | | |
| Total Volume | | 50.0 µL | | | |

2. Mix the reaction well by pipetting or flicking the tube.
3. Spin the tube briefly in a mini centrifuge to collect the liquid.
4. Incubate at 16ºC for 1 hour and then either place on ice or immediately proceed to the next step.

### Post-Ligation Cleanup

1. Incubate at 65ºC for 10 minutes to inactivate the ligase and then place on ice.
2. Centrifuge at 14,000 RCF for 5 minutes in a microcentrifuge. Note the tube orientation in the centrifuge rotor. Transfer the supernatant to a new LoBind tube taking care not to touch the pipette tip to the outer-facing tube wall where any pelleted material may be located. A pellet may be visible on the sidewall of the tube after the supernatant is removed. Discard the tube with pelleted material and place the new tube with supernatant on ice.
3. After centrifugation, pool samples that are to be combined in a multiplexed library.
4. Proceed to the next step.

## AMPure PB Bead Purification of SMRTbell Library

Perform one purification following the **Adapter Ligation** step using 0.45X sample volume of AMPure PB beads.

| STEP | ✓ | Instructions | Notes |
|---|---|---|---|
| **1** | | Add the appropriate volume of AMPure PB beads to the sample based on the specified ratio of beads to sample volume.<br><br>**Note:** Mix the bead reagent well until the solution appears homogeneous before dispensing. Pipette the reagent slowly as the bead mixture is viscous. Precise volumes are critical to the purification process. | |
| **2** | | Mix the solution thoroughly by vortexing or flicking the tube and then spin briefly to collect the liquid. | |
| **3** | | Vortex the tube for 10 minutes at 2,000 rpm to bind DNA to the beads. After vortexing, the solution should appear homogeneous.<br><br>**Note:** We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recovery. | |
| **4** | | Spin the tube briefly to collect the liquid. | |
| **5** | | Collect the beads to the side of the tube in a magnetic bead rack. Allow beads to separate for at least 2 minutes or until the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added. | |
| **6** | | With the tube still on the magnetic bead rack, slowly remove the cleared supernatant and save in another tube. Avoid disturbing the bead pellet.<br><br>**Note:** If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB purification steps to recover the DNA. | |
| **7** | | Wash beads with freshly prepared 80% ethanol.<br>– Do not remove the tube from the magnetic rack.<br>– Slowly dispense the 80% ethanol against the side of the tube opposite the beads, taking care not to disturb the bead pellet. Use a sufficient volume of 80% ethanol to fill the tube (400 µL for 0.5 mL tube or 1 mL for 1.5 mL tube).<br>– After 30 seconds, slowly remove the 80% ethanol and discard.<br><br>**Note:** 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Measure the volumes of water and ethanol separately before combining to ensure the solution is 80% ethanol. 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. | |
| **8** | | Repeat Step 7 above 2 more times for a total of 3 washes. | |

| | | | |
|---|---|---|---|
| **9** | | Remove residual 80% ethanol.<br>– Remove the tube from the magnetic bead rack and spin briefly to pellet the beads. Both the beads and any residual 80% ethanol will be at the bottom of the tube.<br>– Place the tube back on the magnetic bead rack.<br>– Slowly remove any remaining 80% ethanol and discard. | |
| **10** | | Check for any remaining droplets in the tube. If droplets are present, repeat Step 9. | |
| **11** | | Remove the tube from the magnetic bead rack. Allow the beads to air-dry with tube caps open for 30 to 60 seconds. | |
| **12** | | Elute the DNA off the beads.<br>– Add 50 µL Elution Buffer to the beads and mix until homogeneous.<br>– Vortex the tube for 5 minutes at 2,000 rpm.<br>– Spin the tube briefly to collect the liquid and then place the tube back on the magnetic bead rack.<br>– Let the beads separate fully and then without disturbing the bead pellet, carefully transfer the supernatant containing DNA to a new LoBind microcentrifuge tube. Keep the purified DNA on ice.<br>– Discard the beads.<br>– The purified DNA can be stored overnight at 4ºC or at -20ºC for longer duration before proceeding to the next step. | |

**Optional Stop Point**

## Exonuclease Digestion

Failed ligation products and gDNA fragments with treated ends are removed with an exonuclease treatment. Following this, an additional treatment of the SMRTbell library with trypsin facilitates removal of enzymes during the AMPure PB bead purification step.

### Digest Setup

1. To a LoBind microcentrifuge tube, add the following reagents in the order listed:

| Reagent | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|
| SMRTbell Library | | 50.0 µL | | | |
| Water (nuclease-free) | | 38.0 µL | | | |
| CutSmart Buffer | 10X | 10.0 µL | 1X | | |
| Exonuclease III | 100 U/µL | 1.2 µL | 1.2 U/µL | | |
| Exonuclease VII | 10 U/µL | 0.8 µL | 0.08 U/µL | | |
| Total Volume | | 100.0 µL | | | |

2. Mix the reaction well by pipetting or flicking the tube.
3. Spin the tube briefly in a mini centrifuge to collect the liquid.
4. Incubate at 37ºC for 1 hour and then place on ice.
5. Immediately proceed to the next step.

### Trypsin Treatment

1. To the tube from the previous step, add the following reagents in the order listed:

| Reagent | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|
| SMRTbell Library | | 100.0 µL | | | |
| Elution Buffer | | 91.0 µL | | | |
| EDTA | 0.5 M | 5.0 µL | 12.5 mM | | |
| SOLu-Trypsin | 1 mg/mL | 4.0 µL | 20 µg/mL | | |
| Total Volume | | 200.0 µL | | | |

2. Mix the reaction well by pipetting or flicking the tube.
3. Spin the tube briefly in a mini centrifuge to collect the liquid.
4. Incubate at 37ºC for 20 minutes and then place on ice.
5. Immediately proceed to the next step.

## AMPure PB Bead Purification of Exonuclease-Digested SMRTbell Library

Perform two purifications following trypsin treatment, both using 0.45X sample volume of AMPure PB beads.

| STEP | ✓ | Instructions for 1st Purification | Notes |
|---|---|---|---|
| **1** | | Add the appropriate volume of AMPure PB beads to the sample based on the specified ratio of beads to sample volume.<br><br>**Note:** Mix the bead reagent well until the solution appears homogeneous before dispensing. Pipette the reagent slowly as the bead mixture is viscous. Precise volumes are critical to the purification process. | |
| **2** | | Mix the solution thoroughly by vortexing or flicking the tube and then spin briefly to collect the liquid. | |
| **3** | | Vortex the tube for 10 minutes at 2,000 rpm to bind DNA to the beads. After vortexing, the solution should appear homogeneous.<br><br>**Note:** We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recovery. | |
| **4** | | Spin the tube briefly to collect the liquid. | |
| **5** | | Collect the beads to the side of the tube in a magnetic bead rack. Allow beads to separate for at least 2 minutes or until the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added. | |
| **6** | | With the tube still on the magnetic bead rack, slowly remove the cleared supernatant and save in another tube. Avoid disturbing the bead pellet.<br><br>**Note:** If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB purification steps to recover the DNA. | |
| **7** | | Wash beads with freshly prepared 80% ethanol.<br>– Do not remove the tube from the magnetic rack.<br>– Slowly dispense the 80% ethanol against the side of the tube opposite the beads, taking care not to disturb the bead pellet. Use a sufficient volume of 80% ethanol to fill the tube (400 µL for 0.5 mL tube or 1 mL for 1.5 mL tube).<br>– After 30 seconds, slowly remove the 80% ethanol and discard.<br><br>**Note:** 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Measure the volumes of water and ethanol separately before combining to ensure the solution is 80% ethanol. 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. | |
| **8** | | Repeat Step 7 above 2 more times for a total of 3 washes. | |

| | | | |
|---|---|---|---|
| **9** | | Remove residual 80% ethanol.<br>– Remove the tube from the magnetic bead rack and spin briefly to pellet the beads. Both the beads and any residual 80% ethanol will be at the bottom of the tube.<br>– Place the tube back on the magnetic bead rack.<br>– Slowly remove any remaining 80% ethanol and discard. | |
| **10** | | Check for any remaining droplets in the tube. If droplets are present, repeat Step 9. | |
| **11** | | Remove the tube from the magnetic bead rack. Allow the beads to air-dry with tube caps open for 30 to 60 seconds. | |
| **12** | | Elute the DNA off the beads.<br>– Add 50 µL Elution Buffer to the beads and mix until homogeneous.<br>– Vortex the tube for 5 minutes at 2,000 rpm.<br>– Spin the tube briefly to collect the liquid and then place the tube back on the magnetic bead rack.<br>– Let the beads separate fully and then without disturbing the bead pellet, carefully transfer the supernatant containing DNA to a new LoBind microcentrifuge tube. Keep the purified DNA on ice.<br>– Discard the beads.<br>– Proceed to the second AMPure PB bead purification. | |

| STEP | ✓ | Instructions for 2nd Purification | Notes |
|---|---|---|---|
| **1** | | Add the appropriate volume of AMPure PB beads to the sample based on the specified ratio of beads to sample volume.<br><br>**Note:** Mix the bead reagent well until the solution appears homogeneous before dispensing. Pipette the reagent slowly as the bead mixture is viscous. Precise volumes are critical to the purification process. | |
| **2** | | Mix the solution thoroughly by vortexing or flicking the tube and then spin briefly to collect the liquid. | |
| **3** | | Vortex the tube for 10 minutes at 2,000 rpm to bind DNA to the beads. After vortexing, the solution should appear homogeneous.<br><br>**Note:** We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recovery. | |
| **4** | | Spin the tube briefly to collect the liquid. | |
| **5** | | Collect the beads to the side of the tube in a magnetic bead rack. Allow beads to separate for at least 2 minutes or until the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added. | |
| **6** | | With the tube still on the magnetic bead rack, slowly remove the cleared supernatant and save in another tube. Avoid disturbing the bead pellet.<br><br>**Note:** If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB purification steps to recover the DNA. | |
| **7** | | Wash beads with freshly prepared 80% ethanol. | |

| | | | |
|---|---|---|---|
| | | – Do not remove the tube from the magnetic rack.<br>– Slowly dispense the 80% ethanol against the side of the tube opposite the beads, taking care not to disturb the bead pellet. Use a sufficient volume of 80% ethanol to fill the tube (400 µL for 0.5 mL tube or 1 mL for 1.5 mL tube).<br>– After 30 seconds, slowly remove the 80% ethanol and discard.<br><br>**Note:** 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Measure the volumes of water and ethanol separately before combining to ensure the solution is 80% ethanol. 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. | |
| **8** | | Repeat Step 7 above 2 more times for a total of 3 washes. | |
| **9** | | Remove residual 80% ethanol.<br>– Remove the tube from the magnetic bead rack and spin briefly to pellet the beads. Both the beads and any residual 80% ethanol will be at the bottom of the tube.<br>– Place the tube back on the magnetic bead rack.<br>– Slowly remove any remaining 80% ethanol and discard. | |
| **10** | | Check for any remaining droplets in the tube. If droplets are present, repeat Step 9. | |
| **11** | | Remove the tube from the magnetic bead rack. Allow the beads to air-dry with tube caps open for 30 to 60 seconds. | |
| **12** | | Elute the DNA off the beads.<br>– Add 6.8 µL Elution Buffer to the beads and mix until homogeneous.<br>– Vortex the tube for 5 minutes at 2,000 rpm.<br>– Spin the tube briefly to collect the liquid and then place the tube back on the magnetic bead rack.<br>– Let the beads separate fully and then without disturbing the bead pellet, carefully transfer the supernatant containing DNA to a new LoBind microcentrifuge tube. Keep the purified DNA on ice.<br>– Discard the beads. | |
| **13** | | Verify your DNA amount and concentration using a Qubit fluorometer.<br>– Use 0.5 µL of eluted sample to measure the DNA concentration using the Qubit 1X dsDNA HS Assay Kit according to the manufacturer's recommendations.<br>– Record the concentration and amount of recovered sample. Typical DNA yield is between 0.5 to 2% of the starting gDNA input amount.<br>– The purified DNA can be stored overnight at 4ºC or at -20ºC for longer duration before proceeding to the next step. | |

**Optional Stop Point**

## Sample Complex Preparation

Follow instructions below for preparation of sample complex. Sample Setup in SMRT Link cannot be used with this procedure.

**Primer Annealing**

1. Prepare diluted Sequencing Primer v4, before reaction setup, by adding 1 µL primer to 29 µL Elution Buffer.
2. To a 0.2 mL PCR thermal cycling tube, add the following reagents to condition the sequencing primer before annealing to SMRTbell templates:

| Reagent | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|
| 10X Primer Buffer v2 | 10X | 36.0 µL | 6.7X | | |
| Diluted Sequencing Primer v4 | 10X | 18.0 µL | 3.3X | | |
| Total Volume | | 54.0 µL | | | |

3. Mix the reaction well by pipetting or flicking the tube.
4. Spin the tube briefly in a mini centrifuge to collect the liquid.
5. Incubate at 80ºC for 2 minutes and then hold at 4ºC or on ice.
6. Transfer the conditioned sequencing primer to a new LoBind microcentrifuge tube and keep on ice until ready to use.

   **Note:** Any remaining conditioned sequencing primer may be stored at -20°C and used for up to 30 days.
7. To a new 0.2 mL PCR thermal cycling tube, add the following reagents to anneal conditioned sequencing primer to SMRTbell templates:

| Reagent | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|
| Conditioned Sequencing Primer v4 | 3.3X | 2.7 µL | 1X | | |
| SMRTbell Library | | 6.3 µL | | | |
| Total Volume | | 9.0 µL | | | |

8. Mix the reaction well by pipetting or flicking the tube.
9. Spin the tube briefly in a mini centrifuge to collect the liquid.
10. Incubate at 20ºC for 1 hour and then hold at 4ºC or on ice.
11. Proceed to the next step.

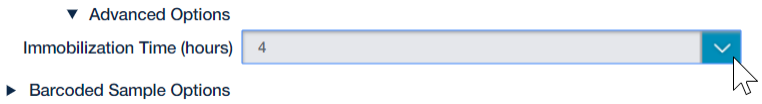
**Polymerase Binding**

1. Prepare diluted Sequel DNA Polymerase 3.0 just prior to use by adding 1 µL polymerase stock to 29 µL Sequel Binding Buffer and keep on ice. Diluted polymerase must be used immediately. Discard the unused portion.
2. To the PCR thermal cycling tube containing primer-annealed SMRTbell library, add the following reagents in the order listed:

| Reagent | Stock Conc. | Volume | Final Conc. | ✓ | Notes |
|---|---|---|---|---|---|
| SMRTbell Library with annealed primer from previous step | | 9.0 µL | | | |
| Sequel Binding Buffer | 10X | 1.5 µL | 1X | | |
| DTT | 10X | 1.5 µL | 1X | | |
| Sequel dNTP | 10X | 1.5 µL | 1X | | |
| **Mix before proceeding** | | | | | |
| Diluted Sequel DNA Polymerase 3.0 | 10X | 1.5 µL | 1X | | |
| Total Volume | | 15.0 µL | | | |

3. Mix the reaction well by pipetting or flicking the tube.
4. Spin the tube briefly in a mini centrifuge to collect the liquid.
5. Incubate at 30ºC for 4 hours and then hold at 4ºC or on ice until ready for sample complex purification.

## Sample Complex Purification

| STEP | ✓ | Instructions | Notes |
|---|---|---|---|
| 1 | | To a 1.5 mL LoBind microcentrifuge tube, add 35 µL Sequel Complex Dilution Buffer then add 15 µL of sample complex. | |
| 2 | | Add 30 µL AMPure PB beads (0.6X sample volume) to the tube containing 50 µL diluted sample complex. | |
| 3 | | Mix well by pipetting or flicking the tube, spin, and incubate for 5 minutes on the benchtop to bind sample to the beads.<br>**Note:** Longer incubation times have not been tested and may have a negative impact on polymerase-template complex stability due to high salt concentration. | |
| 4 | | Collect the beads to the side of the tube in a magnetic bead rack. Allow the beads to separate for 2 minutes or until the solution appears clear and then slowly remove the supernatant and discard.<br>**Note: DO NOT wash the collected bead pellet with ethanol.** | |
| 5 | | Remove the tube from the magnetic bead rack and spin briefly to pellet the beads. Place the tube back on the magnetic bead rack. Slowly remove any remaining supernatant and discard. | |
| 6 | | Immediately resuspend the beads in 74.5 µL room temperature Sequel Complex Dilution Buffer. | |
| 7 | | Mix well by pipetting or flicking the tube, spin, and incubate at room temperature for 15 minutes to elute sample from the beads. | |
| 8 | | Collect the beads to the side of the tube in a magnetic bead rack. Allow the beads to separate for 1 minute or until the solution appears clear and then transfer the supernatant to a new LoBind microcentrifuge tube. Keep the tube with sample on ice. | |
| 9 | | Perform two 100-fold serial dilutions of Sequel DNA Internal Control Complex 3.0 in Sequel Complex Dilution Buffer. Dilute the final serial dilution 2-fold in Sequel Complex Dilution Buffer for a final 20,000-fold dilution. Keep all dilutions on ice. | |
| 10 | | To the tube containing eluted sample, add 1.0 µL of the 20,000-fold dilution of internal control complex, 8.5 µL DTT, and 1.0 µL Sequel Additive. | |
| 11 | | Mix well by pipetting or flicking the tube, spin, and transfer the sample to a sample plate. | |
| 12 | | Cover the sample plate and keep at 4ºC or on ice until ready to sequence. | |
| 13 | | Sequence with diffusion loading, an immobilization time of 4 hours, a pre-extension time of 1 hour, and a movie time of 20 hours.<br>To specify a custom 4-hour immobilization time, go to the Advanced Options section in SMRT Link Run Design and select 4 hours from the Immobilization Time drop-list menu.<br>  | |

| Revision History (Description) | Version | Date |
|---|---|---|
| Initial Release. | 01 | August 2019 |
| Updated recommendation to use Qubit 1X dsDNA HS Assay Kit. Updates to Reagent Preparation section on page 9, AMPure PB bead Purification on page 15, Adapter Ligation on page 16 (reduced barcoded adapter volume from 10 µL to 1 µL), and Sample Complex Purification on page 25 (increased movie time from 10 hours to 20 hours). | 02 | September 2019 |

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